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Structure of 7,12-dimethylbenz[*a*]anthracene-guanosine adducts*

(polycyclic aromatic hydrocarbons/carcinogens/arene oxides/nucleic acids)

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ABSTRACT **Arene oxides have been proposed as the reactive intermediates in the process of carcinogenesis induced by polycyclic aromatic hydrocarbons. The present study defines the structures of four guanosine adducts formed by the reaction of 7,12-dimethylbenz[*a*]anthracene-5,6-oxide with polyguanylic acid. The modified polymer was hydrolyzed to nucleotides and the hydrophobic guanosine adducts separated from unmodified guanosine by LH-20 column chromatography. The adducts were further resolved into four components (I–IV) by reverse phase high pressure liquid chromatography. Analysis of the ultraviolet, circular dichroism, mass, and proton magnetic resonance spectra of these compounds, or their acetate and free base derivatives, indicates that in all four compounds the aromatic hydrocarbon is present on the 2 amino group of guanine. Compounds I and IV, and II and III constitute diastereoisomeric pairs, respectively. In the I and IV pair, the adducts result from addition at the 6 position of the original dimethylbenz[*a*]anthracene oxide, whereas in the II and III pair, the addition occurs at the 5 position. Indirect evidence suggests that *trans* opening of the oxide occurred in all cases but this remains to be established.**

The covalent binding of chemical carcinogens to cellular macromolecules, and in particular, to nucleic acids, is considered to be an essential aspect in their action (1). Several carcinogens, including polycyclic aromatic hydrocarbons, require metabolic activation before covalent binding occurs. Arene oxides have been proposed as the reactive intermediates for the polycyclic aromatic hydrocarbons (2), and evidence has been presented to support this theory (3, 4). Previous studies demonstrated that the K region oxide of 7,12-dimethylbenz[*a*]anthracene (DMBA), DMBA-5,6-oxide, reacts *in vitro* preferentially with guanine residues in nucleic acids and evidence for the formation of more than one covalent adducts was obtained (5). More recent studies on the enzyme-mediated binding of DMBA to poly(G) in a microsomal system indicate the formation of a large number of guanosine adducts some of which appear to be the same as those formed in the direct reaction of DMBA-5,6-oxide with poly(G) (6). The K region oxides of DMBA, 7-methylbenz[*a*]anthracene, and benzo[*a*]pyrene have a number of biological activities on cells in culture including cytotoxicity, mutagenicity, and cell transformation (7–10). With benzo[*a*]pyrene (11) and benz[*a*]anthracene (12) however, there is evidence that a diol-epoxide rather than K region oxide is the major metabolite involved in nucleic acid binding *in vivo*. In the case of DMBA the *in vivo* intermediate has not been identified.

Abbreviations: DMBA, 7,12-dimethylbenz[*a*]anthracene; DMBA-nucleoside, the guanosine adduct formed after reaction of poly(G) with DMBA-5,6 oxide (in this case the DMBA does not represent the parent hydrocarbon itself); HPLC, high pressure liquid chromatography; CD, circular dichroism; PMR, proton magnetic resonance; *m/e*, mass/electron.

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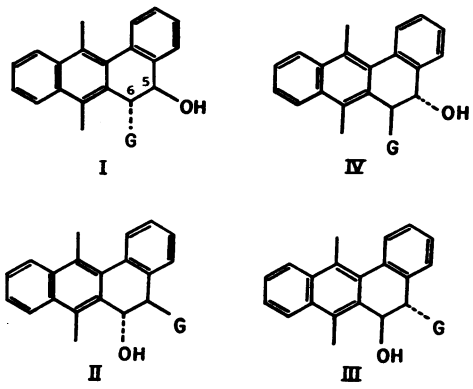
Although considerable effort has been expended on studies of the metabolism of the polycyclic aromatic hydrocarbons, no information is available on the actual structures of the nucleic acid adducts. This type of information might provide clues to activation mechanisms and insights into the process by which these carcinogens modify the structure and function of cellular nucleic acids. In the present study we have elucidated the chemical structure of the guanosine adducts I–IV formed from



the *in vitro* reaction of DMBA-5,6-oxide with poly(G). Our results provide the structures of polycyclic aromatic hydrocarbon adducts formed by the reaction of nucleic acids with an arene oxide.

METHODS AND MATERIALS

Details of the reaction of nucleic acids with DMBA-5,6-oxide have been previously described (5). Poly(G) was treated with the oxide in 50% acetone in water after which the unbound material was removed by repeated solvent extractions and precipitations of the nucleic acid with ethanol. After hydrolysis of the modified polymer, the modified nucleotides were separated from GMP by Sephadex LH-20 column chromatography and then treated with alkaline phosphatase. Further fractionation of the modified nucleosides by high pressure liquid chromatography (HPLC) led to the isolation of four distinct DMBA-guanosine adducts: I–IV. HPLC was done on a Dupont 830 instrument with reverse phase Zorbax ODS columns operated at 50° and 3000 psi (20.68 MPa) and eluted with methanol–water. Additional details are described elsewhere (6). Nucleoside adducts were converted to their corresponding bases by treatment with periodate and lysine (13). The nucleoside adducts or their corresponding bases were acetylated with acetic anhydride in pyridine at room temperature for 6 hr and the solvents removed under reduced pressure before re-purification by HPLC. The proton magnetic resonance (PMR) data on the DMBA-guanine adducts and their acetates were obtained on racemic mixtures prepared by mixing the diastereoisomeric nucleosides (I + IV and II + III), converting each pair to their corresponding DL-bases with periodate-lysine, purification by

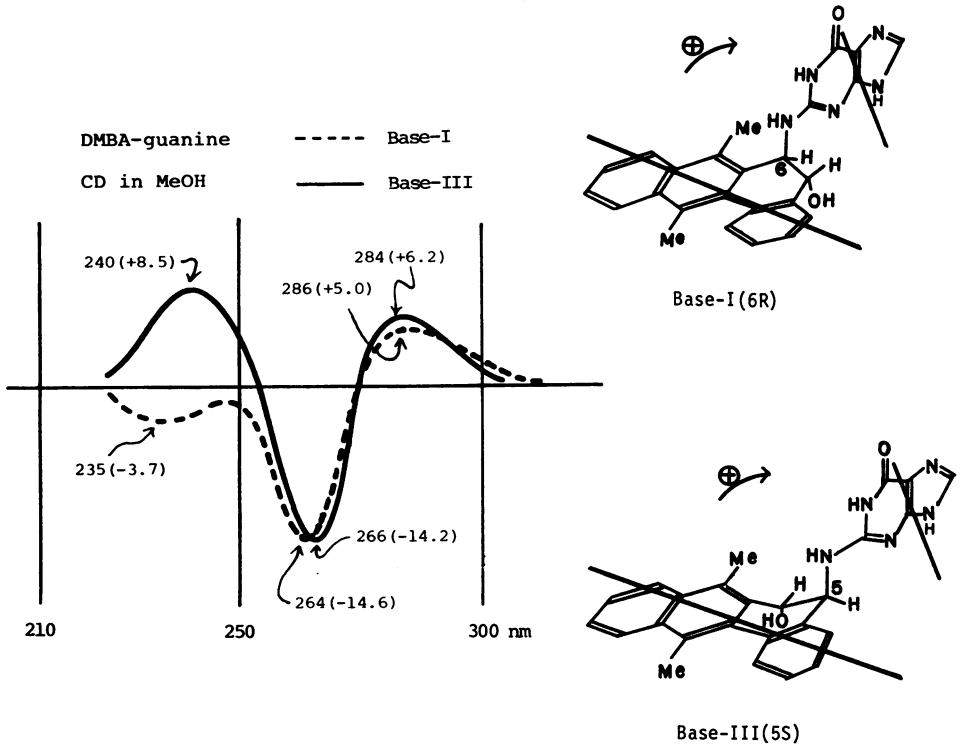


FIG. 1. CD spectra (in methanol) of bases I and III obtained from DMBA-nucleoside adducts I and III, respectively. The CD spectrum of base IV ($\Delta\epsilon_{286} - 4.7$, $\Delta\epsilon_{263} + 14.0$, $\Delta\epsilon_{236} + 4.3$) was antipodal to that of base I, whereas that of base II ($\Delta\epsilon_{284} - 6.4$, $\Delta\epsilon_{266} + 14.7$, $\Delta\epsilon_{242} - 9.5$) was antipodal to that of base III.

HPLC, acetylation, and repurification by HPLC. The diastereoisomeric nucleosides were combined because of scarcity of material. UV spectra were obtained in methanol on a Cary model 14 recording spectrophotometer, circular dichroism (CD) spectra in methanol on a Jasco J-40 instrument and PMR spectra at 100 MHz on a Jeol PS-100 instrument in 99.5% methanol-d_4 (totally deuterated methanol), after exchange with the same solvent. Mass spectra were obtained on MS9 or Finnigan 3300 instruments.

DMBA-5,6-oxide was synthesized and characterized as previously described (14). Poly(G) was purchased from Schwarz/Mann and triacetylguanosine from Sigma Chemical Co.

RESULTS

From the modification of 100 μmol of nucleotide as poly(G) with 100 μmol of DMBA-5,6-oxide, 10 μmol of DMBA-guanosine derivatives were obtained after chromatography on LH-20 Sephadex. These were resolved into four major products by HPLC (6), utilizing a Zorbax ODS column eluted with 40% methanol at 50°. The compounds, designated in order of elution, had the following retention times and % recoveries of applied material (based on UV absorption): I, 34 min, 15%; II, 37 min, 28%; III, 37.5 min, 28%; and IV, 39 min, 21%. Subsequent analysis indicated that compound I was pure and that II, III, and IV was each contaminated with its predecessor by less than 6%. The UV spectra of these compounds in methanol were all identical, with λ max at 260 nm and absorption extending to about 330 nm, and were the same as the previously described "type II' product (5). The spectra closely resembled that of DMBA-5,6-dihydrodiols but were dissimilar from the parent hydrocarbon, suggesting a 5,6-dihydro-DMBA structure for the adducts. The contribution to the spectrum of the guanosine moiety was largely obscured by that of the hydrocarbon nucleus.

A critical finding which greatly simplified the structural studies was that the CD spectra of nucleoside adducts I and IV and of adducts II and III, respectively, indicated a mirror image relationship (the CD spectra were not exact mirror images because the D-ribose unit was common to all the adducts). Similarly the CD spectra of the bases I and IV, and II and III constituted mirror image pairs (Fig. 1). The optical activity of the four bases provided further evidence that they were 5,6-dihydro-DMBA derivatives; fully aromatic DMBA derivatives would have been optically inactive.

Mass spectra of compounds I–IV could not be obtained by chemical ionization. Mass spectra obtained by electron impact at either 20 or 70 eV failed to reveal the molecular ion, a behavior which is typical for guanosine derivatives (15, 16), although each compound showed very strong ions at mass/electron (m/e) 256 and 271 (Fig. 2). In addition, ions were observed for each compound at 151 (guanine) and 405. The ions at 405 correspond to the loss of water and ribose from a simple adduct between guanosine and DMBA-oxide. The ions at 271 were particularly diagnostic since these could result from dehydration across the 5,6-bond of DMBA and cleavage of the C-2 and N^2 bond of guanosine, suggesting that the adducts were linked through the N^2 group. The ions at m/e 271 of compounds I and III matched well to $C_{20}H_{17}N$, both results being 271.1357 (271.1361 theory) (see Fig. 2). At high resolution, the 256 ion of compound III appeared as a doublet with values of 256.1233 (calculated for $C_{20}H_{16}$: 256.1252) and 256.1152 (calculated for $C_{19}H_{14}N$: 256.1126), corresponding to losses of NH and CH_3, respectively, from the 271 fragment. For compound I the 256 doublet was poorly resolved, the average value being 256.1177.

A similar cleavage of the N^2 to C-2 bond has been observed with N^2-methylguanine although in that case the charge remains on the purine residue (17). Other positions of substitution on the purine ring (including O-6, N-7, or C-8) would not be

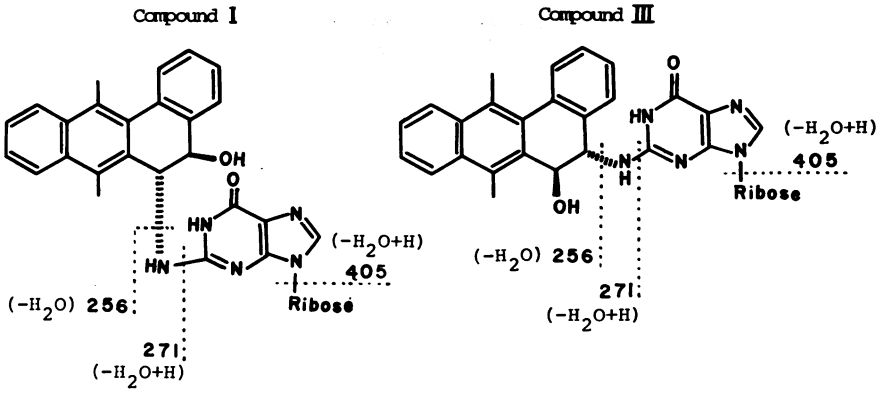


FIG. 2. Mass fragmentations of Compounds I and III.

expected to give ions corresponding to the observed formula of $C_{20}H_{17}N$.

Attempts were made to prepare more volatile derivatives of the nucleoside adducts by acetylation. The products were separated by HPLC on a Zorbax ODS column eluted with 65% methanol at 50°. The retention times for each of the compounds were: I, 16 min; IV, 18 min; II, 24 min; and III, 26 min. Since the order of elution changed after acetylation and the diastereoisomeric pairs eluted with very similar retention times, it appears that intramolecular hydrogen bonding is important in the reverse phase separation of these DMBA-nucleoside adducts. Again, no useful spectra of these acetates were obtained by chemical ionization mass spectrometry. With electron impact ionization, compound IV acetate showed weak, but distinct ions corresponding to m/e 405, 663, 705, and 723 (the tetraacetate molecular ion), the ratios of which remained constant during heating of the probe suggesting that they all arose from a single component. The acetate of compound II showed a similar spectrum although the molecular ion could not be detected. The apparent loss of water (m/e 705) from the molecular ion of the acetates of compounds II and IV was attributed to the intermolecular acetyl transfer from O to N to give a pentaacetate in the mass spectrometer, followed by loss of acetic acid. This is more likely than direct dehydration, since under the conditions used for acetylation no N-acetylation should have occurred (18). Evidence for intermolecular transfer of an acetyl residue was obtained when a sample of triacetylguanosine, which was judged pure by HPLC and which showed only three acetyl groups in its PMR spectrum in dimethylsulfoxide-d_6, gave an ion at m/e 451 corresponding to tetraacetyl guanosine (8% base peak) in addition to an ion of equal intensity at 409. Similarly ions at m/e 452 and 410 were obtained by chemical ionization of triacetylguanosine. These ions retained a constant ratio during heating of the probe suggesting the presence of a single component.

The PMR data of compounds I–IV and derivatives are summarized in Table 1; Fig. 3 is a representative spectrum. A slightly broadened singlet was observed in the aromatic region of the spectrum of compound I, at δ 8.03 (Fig. 3). The corresponding values for II–IV were 8.00, 7.92, 7.98, respectively. These were assigned to the C-8 proton of guanine since the aromatic protons of the DMBA moiety all show strong *ortho* coupling. We can also conclude that substitution has not occurred on the N-7 position of guanine since this would cause the proton on carbon atom 8 to be much further downfield because of the positive charge on the imidazole ring (19). The

Table 1. PMR spectra of DMBA derivatives:
Chemical shifts of benzylic and methyl protons in CD_3OD

Entry	Compounds	Benzylic protons (5-H and 6-H)	Methyl protons
1	I	5.82 (6H) 5.07 (5H)	2.97 2.74
2	IV	5.84 (6H) 5.09 (5H)	2.98 2.75
3	II	5.51 (6H) 5.43 (5H)	2.97 2.77
4	III	5.50 (6H) 5.40 (5H)	2.98 2.76
5	DMBA-5,6-dihydro-*trans*-diol	5.20 (6H) 4.74 (5H)	2.93 (12Me) 2.82 (7Me)
6	Base I + IV	5.84 (6H) 5.05 (5H) Δ = 0.64	2.99 2.76
7	Base I + IV Acetate	6.16 (5H) 5.80 (6H) Δ = 0.31 Δ = 0.29	2.96 2.70
8	Base II + III	5.49 (6H) 5.38 (5H) Δ = 0.64	3.00 2.80
9	Base II + III Acetate	6.75 (6H) 5.45 (5H)	2.98 2.70

7.0 6.5 6.0 5.5 5.0 3.5 3.0 ppm

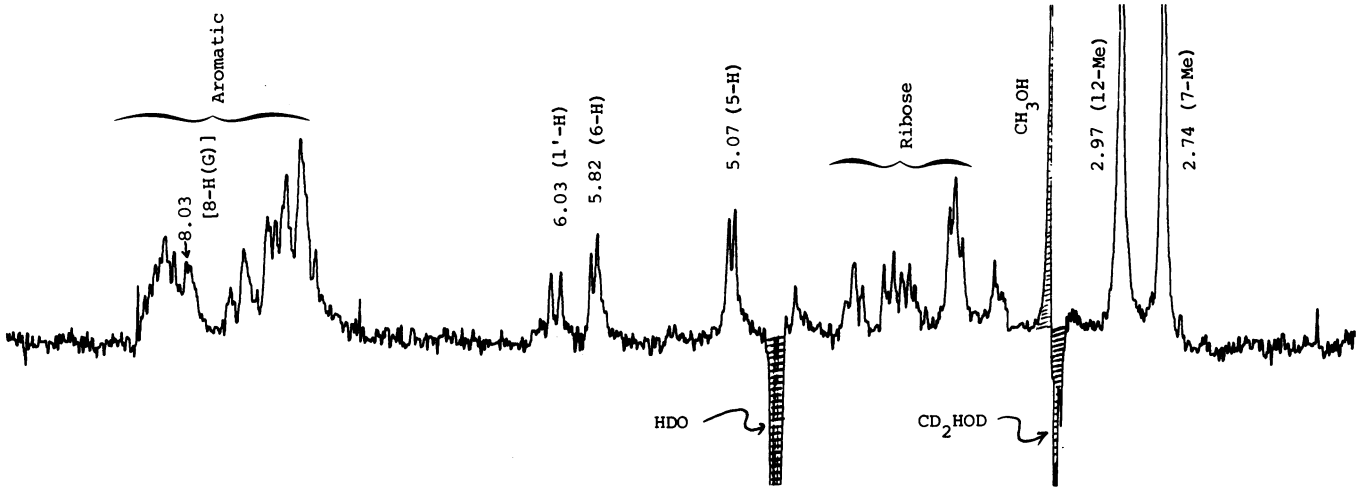


FIG. 3. PMR spectrum of Compound I in CD_3OD as measured by the inversion-recovery sequence, $(180\text{-}\tau\text{-}90\text{-}T)_n$-, where τ was 1.2 sec and T 6.6 sec, 2048 scans.

PMR data indicating that substitution has not occurred on the C-8 or N-7 of guanine agree with the above mass spectral data.

Analysis of the chemical shifts of the benzylic protons of compounds I–IV was made in comparison with *trans* DMBA-5,6-dihydrodiol (Table 1). The assignments of the latter were previously based on theoretical considerations and comparisons with other compounds (14). This has now been confirmed directly by nuclear Overhauser enhancement experiments, in which a 10.6% enhancement of the 5.20 ppm benzylic proton was obtained by irradiation of the upfield methyl group. The downfield 5.20 ppm signal thus is due to 6-H. We assumed that substitution of the guanine on C-5 and C-6 will: (*i*) displace the 5-H and 6-H signals, respectively, to approximately the same extent and (*ii*) displace the adjacent 6-H and 5-H signals also to approximately the same extent. However, the displacements observed in (*i*) and (*ii*) may not be equal. We then arrived at the assignments shown in Table 1, where one of the methines is shifted by 0.31 ppm (5-H in entry 6) and 0.29 ppm (6-H in entry 8), whereas the other methine is shifted by 0.64 ppm (6-H in entry 6 and 5-H in entry 8). In the I + IV acetate prepared from bases I + IV (entry 7), the 5-H is shifted downfield by 1.11 ppm (from 5.05 to 6.16 ppm) while the chemical shift of 6-H remains unchanged; accordingly, in compounds I and IV the guanosine is substituted on C-6. In compounds II and III the guanosine is substituted on C-5 since a 1.26 ppm downfield shift of 6-H (entry 9) occurs upon acetylation of bases II + III. When the nucleosides I and IV, or II and III had been mixed and converted to their corresponding bases or subsequently acetylated, they appeared in each case to be single compounds by PMR spectroscopy; this supported the conclusion from the CD spectra that these compounds were diastereoisomeric pairs.

The *cis* and *trans* DMBA-5,6-dihydrodiols and all four of the adducts had small J_{5-6} coupling constants in the range 3.2 ± 0.5 Hz corresponding to ea or ee oriented protons. It was, therefore, not possible to distinguish between the *cis* and *trans* configurations from the PMR data. Although *trans* substitution is more likely, *cis* openings of arene oxides have been described (20, 21). During the modification of poly(G) we have observed, in addition to the preponderant *trans* hydration of DMBA-5,6-oxide, a small amount of the *cis* dihydrodiol in the ether extracts. In recent studies on the reaction of sulfur nucleophiles with K-region oxides (28), analysis of the PMR coupling constants of the products in various solvents indicated that they resulted from *trans* addition. In the case of compounds I–IV, we assumed that *trans* openings of the oxide had occurred on general mechanistic grounds and also in view of the steric bulk of the guanosine moiety, but this remains to be established.

DMBA-5,6-oxide and the DMBA-GMP adducts both have a strong absorption band around 270 nm (6). This can provisionally be assigned to the longitudinal ^1B_b transition of the naphthalene chromophore which has been displaced by the 2-phenyl substituent (see Fig. 1). The 270 nm band of GMP is due to the transverse transition moment (23), and hence it is tempting to interpret the split Cotton effects centered around 275 nm in bases I and III (Fig. 1) as being caused by coupled oscillators (24–26 and references cited therein). The absolute configurations depicted in Fig. 1 for compounds I and III are derived on this basis. They are tentative until a more rigorous theoretical treatment has been carried out.¶

DISCUSSION

The present results indicate that the reaction of the K region oxide of DMBA with poly(G) leads to the formation of four distinct nucleoside adducts. In all four adducts, the polycyclic aromatic hydrocarbon is linked to the 2 amino group of guanine. Compounds I and IV constitute a diastereoisomeric pair in which the 2 amino group is linked to the 6 position of 5-hydroxy-5,6-dihydro DMBA; compounds II and III are another diastereoisomeric pair linked to the 5 position of 6-hydroxy-5,6-dihydro DMBA. Indirect evidence suggests that in all four cases the guanine residue is *trans* to the adjacent hydroxyl.

Electronic factors would predict that nucleophilic attack is more likely to occur on the 6 position of DMBA-5,6-oxide (27), and this is the case with *t*-butylmercaptan (28). With poly(G), however, the proportions of 5 and 6 substituted products were about equal, perhaps reflecting the local environment of the amino groups in poly(G) under the conditions used for reaction. It is interesting that 7-bromomethylbenz[*a*]anthracene also binds to the 2-amino group of guanosine in nucleic acids (29). Acetylaminofluorene binds predominantly to the C-8 of guanosine but there appears to be some binding also to the 2 amino group (30).

The reactivity of the amino group of guanine with an arene oxide contrasts with results indicating that alkene oxides attack mainly the N-7 position of guanine (19, 22). Guanosine mo-

¶ The direction of the electric transition moment is being measured by Prof. J. Michl, Univ. of Utah.

nonucleotide can be used in place of poly(G) as a substrate for reaction with DMBA-oxide (5, 6), but the products formed appear to be different from those obtained with poly(G) (ref. 6, and Frenkel and Grunberger unpublished studies).

The results we have obtained with poly(G) are not peculiar to this synthetic nucleic acid since the reaction of tRNA with DMBA-5,6-oxide leads to the formation of four guanosine adducts which are identical on HPLC to the compounds obtained from the reaction with poly(G) (6). In addition to the guanosine adducts, DMBA-5,6-oxide forms lesser but appreciable amounts of at least two adenosine adducts when the arene oxide is reacted with either poly(A) or tRNA (5, 6). In view of the results obtained with poly(G), it is likely that this involves reaction of the arene oxide with the 6-amino group of adenosine, but these structures remain to be elucidated.

From molecular models it would appear that the reaction of DMBA-5,6-oxide with the 2 amino group of guanine residues in DNA could occur, without major distortions of the native structure, by insertion of the polycyclic aromatic hydrocarbon into the minor groove of the double helix. This is in contrast to the attack of N-2-acetylaminofluorene at the C-8 position of guanine in which there is steric hindrance and "base displacement" occurs (31–34).

As mentioned in the introduction, there is evidence that *in vivo* the K region oxides of benz[*a*]anthracene and benzo[*a*]pyrene may not be the major metabolites involved in nucleic acid binding. We do not believe, however, that a biological role for K region oxides has been completely excluded. In addition, the methods, approaches, and results described in the present study are likely to apply to the characterization of other adducts formed between polycyclic aromatic hydrocarbon carcinogens and nucleic acids. This structural information should contribute to our understanding of the mechanism of action of this important class of environmental carcinogens.

Note Added in Proof. The *cis* and *trans* DMBA-dihydrodiol diacetates could be distinguished by the chemical shift differences of the acetate protons (14). The *trans* isomer exhibited a single peak at δ 1.84, whereas the *cis* isomer had acetate proton resonances at δ 1.80 and 2.18. The δ 1.80 signal was assigned to the acetate group substituted on C-6, since this group, like both acetate groups of the *trans* isomer, is expected to be oriented axially to minimize steric interaction with the adjacent 7-methyl group. As a consequence, the acetate group substituted on C-5 of the *cis* isomer is forced into the equatorial conformation. The downfield shift of the 5-acetoxy proton signal of the *cis* isomer is consistent with previous observations of other *cis* and *trans* 5- and 6-acetoxy derivatives of 5,6-dihydro-DMBA [ref. 14, and P. Dansette and D. M. Jerina (1974) *J. Am. Chem. Soc.*, **96**, 1224–1225]. Further analysis of the PMR data in the present study indicates that the acetate protons of the acetates of bases I and IV are at δ 1.82, indicating that the acetates are axial. This supports the tentative *trans* assignment of these two guanine adducts.

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